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Building-Associated Neurological Damage Modeled in Human Cells: A Mechanism of Neurotoxic Effects by Exposure to Mycotoxins in the Indoor Environment

Enusha Karunasena · Michael D. Larrañaga · Jan S. Simoni · David R. Douglas · David C. Straus

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Abstract Damage to human neurological system cells resulting from exposure to mycotoxins confirms a previously controversial public health threat for occupants of water-damaged buildings. Leading scientific organizations disagree about the ability of inhaled mycotoxins in the indoor environment to cause adverse human health effects. Damage to the neurological system can result from exposure to trichothecene mycotoxins in the indoor environment. This study demonstrates that neurological system cell damage can occur from satratoxin H exposure to neurological cells at exposure levels that can be found in water-damaged buildings contaminated with fungal growth. The constant activation of inflammatory and apoptotic pathways at low levels of exposure in human brain capillary endothelial cells, astrocytes, and neural progenitor cells may amplify devastation to neurological tissues and lead to neurological system cell damage from indirect events triggered by the presence of trichothecenes.

Keywords Sick-building syndrome · Mycotoxins · Indoor-air quality · Trichothecenes · *Stachybotrys chartarum* · Satratoxin · Neurological damage

E. Karunasena (✉)
Department of Animal & Food Sciences, Texas Tech University, Lubbock, TX 79409, USA
e-mail: enusha.karunasena@ttuhsc.edu

M. D. Larrañaga
School of Fire Protection & Safety Engineering Technology, Oklahoma State University, Stillwater, OK 74078, USA

J. S. Simoni
Department of Surgery, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

D. R. Douglas · D. C. Straus
Department of Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

Introduction

Thousands of buildings suffered water damage and resultant fungal growth in the aftermath of hurricanes Katrina and Rita along the gulf coast of the United States [1]. Potential exposures to fungi and their metabolites present a significant health risk to residents and workers returning to these buildings [1, 2]. Devastation caused by the hurricanes highlighted the public health dilemma facing all industrialized nations from indoor fungal contamination which may lead to sick-building syndrome (SBS) and as evidenced, increased fungal particles potentially carrying mycotoxins [3–5].

The SBS phenomenon began after the oil embargo by the Organization of Petroleum Exporting Countries (OPEC) in the early 1970s. The effects of the embargo prompted the tightening of design and construction for buildings to reduce energy consumed

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in cooling and heating [6]. The tightening of building systems helped seal the indoor environment from the outdoor environment, leading to variations in the fungal spore concentrations indoors relative to outside air [6]. The tightening of building systems decreased the ability of buildings to exchange moisture with the environment. This allowed even minor water leaks from building imperfections, or poorly designed, operated, or maintained heating, ventilating, and air conditioning systems to result in indoor fungal growth [3, 7].

SBS was associated with poor indoor environmental quality (IEQ) in 1982, and the World Health Organization (WHO) made the first attempt to define SBS in the early 1980s [8–10]. Common symptoms included nervous system effects (headaches, lethargy, and fatigue) and mucous membrane (eye, nose, and throat) irritation [8]. No single SBS definition exists, but most researchers agree that SBS describes a number of symptoms that have no clear etiology and are attributable to exposures from within a particular building [8, 11]. As such, the definition of SBS is continuously evolving.

Today, Americans spend about 90% of their time in the indoor environment [12]. Epidemiological studies conducted at office buildings contaminated with *Stachybotrys chartarum* demonstrated abnormal respiratory activity, elevated white blood cell counts, and central nervous system (CNS) disorders [3, 13–17]. Many of the filamentous fungi growing indoors generally produce chemical compounds known as mycotoxins [4]. *Stachybotrys* species are some of the many mycotoxin-producing filamentous fungi that proliferate in damp indoor environments [7, 16, 18–24]. Continued research efforts demonstrate that particle sizes smaller than spores are hundred times more pronounced in a given area and could carry mycotoxins leading to SBS symptoms [25–29]. These studies demonstrate that after prolonged periods of water damage, fungal colonization of building materialscan lead to the dispersion of particles that are undetectable with conventional sampling methods [28, 30]. These fine particles are released through vibrations and air turbulence contributing especially to fungal fragments being aerosolized and materials like ceiling tile particles [25, 28, 31]. Studies conducted in our laboratory found that concentrations of fragments and particles greatly outnumber intact fungal spore concentrations and that mycotoxins are found on both spores and fungal fragments [29, 32]. Seo et al. [25] demonstrate that with age, fungal particles from *S. chartarum* growth on ceiling tile and gypsum board are significantly higher in aerosolized particles and size. Additionally, more fungal fragments than spores may be deposited in the respiratory tract in water-damaged buildings, leading WHO to propose that both spores and fungal fragments may be involved in adverse health effects due to exposure to fungi and their metabolites [33]. SBS symptoms resulting from exposure to fungi and fungal metabolites in water-damaged buildings continues to be a major public health threat in the industrialized nations. In 2008 and 2009, the US Government Accounting Office and the WHO, respectively, published reports on indoor fungal contamination calling for increased research in the health effects due to exposure to fungal particles and mycotoxins in the indoor environment [33, 34].

Biological assays to determine the contribution of fine particles contributing to physiological responses demonstrate both oxidative and inflammatory responses. SBS conditions contribute to chronic upper and lower respiratory disease, and recent studies demonstrate elevated expression of inflammatory makers, intracellular adhesion molecule (ICAM), fibrinogen, and lag4 in environments with high air pollution [27]. Studies conducted on fine particle translocation in an in vivo Apo E-/- murine model demonstrate macrophage (which includes astrocyte and dendritic cells) are unable to phagocytize particles below 0.2 μm [26, 30]. Additionally, smaller particles were able to translocate across lung or olfactory epithelial tissues, compromising these barrier and allowing particles of 0.02 μm to enter systemic circulation through carriage in erythrocytes [26]. Further analysis demonstrated increase markers of oxidative stress, including compromise of blood–brain barrier, neurofibrillary tangles, and increase NF-κB [26].

Exposure to mycotoxins has been shown to result in a decrease in hematopoiesis and symptoms associated with mycotoxin exposure include inflammation of the skin, diarrhea, hemorrhage, emesis, and nervous system abnormalities [35–37]. Mycotoxins are fungal metabolites known to be harmful toward human and animal health [6, 7, 18–24, 35–41]. Building occupants, who claim neurological

symptoms due to SBS, are often viewed with suspicion. This increases difficulty the scientific community has had in establishing legitimate psychological and physiological analysis as evidence of neurological damage due to inhaled mycotoxins [42, 43]. Students exposed to poor IEQ from fungal contamination demonstrated acoustic mycotic neuroma [42]. Although this syndrome is normally seen in elderly populations, adolescent students were found to have high incidences of the condition [42]. These students exhibited other symptoms associated with neurological damage from SBS conditions. The symptoms included headaches, memory loss, lack of concentration, fatigue, sleep disturbances, facial swelling, rashes, nosebleeds, diarrhea, abdominal pains, and respiratory problems [42].

Symptoms due to fungal contaminants are demonstrated to be associated with both inflammation and oxidative stress [13, 14, 17, 30, 44]. Satratoxins and other trichothecenes can increase the production of TNF-α, IL-6, IL-8, IL-2, IL-5, IL-1β, and Fas ligand expression, which are able to activate inflammation [26, 35, 36, 45]. The process of inflammation is intended to repair injured tissues; however, this mechanism tends to induce damage to the CNS when activated [13, 14, 26, 44]. Studies conducted by Calderon-Garciduenas et al. [13, 14] demonstrate increased iNOS, NF-κB, and TNF-α production among other inflammatory and oxidative stress agents—leading to permanent damage of DNA and CNS tissues due to passage of small particles via olfactory epithelium and lung tissue. The fine particles reach CNS tissue via the olfactory bulb and into brain tissues (frontal cortex and cortical tissues) demonstrating increases in β-amyloid plaques suggestive of pathogenesis similar to Alzheimer's disease [13, 14]. Equally, studies reviewed by Thrasher et al. [30] describe the dynamics of indoor air pollution that lead to both growth and neurological defects including studies conducted on aflatoxins and satratoxin G have demonstrated the ability of these compounds to conjugate with albumin—leading to growth impairments in children due to alterations in glutathione S-transferase activity. Ingestion of fumonisin toxins by Mexican and Texas populations, through contaminated corn tortillas has been linked to neural tube defects and fetal death, further demonstrating neurological disease due to mycotoxin exposure [30].

The purpose of this study was to identify the mechanisms, in human neurological system cells, that induce cell damage from exposure to the trichothecene mycotoxin satratoxin H (SH). Through the course of this study, immunological and apoptotic events were evaluated to identify the cellular pathways that were disrupted by exposure to SH. Three human neurological cell lines were utilized in vitro to examine the toxic effects of SH at low to moderate doses Low doses analogous to concentrations found in the air of buildings exhibiting SBS conditions were established through analysis of serial concentrations of SH on human brain capillary endothelial cells (HBCEC) [29]. These concentrations were then used for further examination of cellular events [31]. The cell-systems in this study form the fundamental neural tissues: HBCEC, which form the structure of the blood brain barrier (BBB); astrocytes, which act as macrophages in neural tissues, and neural progenitor cells, or neurons [46]. Neural cells are unable to repair extensive cellular damage from cytotoxic events that induce apoptosis or severe inflammation. Thus, the objective of these experiments was to evaluate the effects exposure to SH would have on cellular homeostasis and to determine whether a model of the BBB could be compromised and neural cell damage could be induced by exposure to macrocyclic trichothecenes at low concentrations that may be present in buildings with poor IEQ as determined through ELISA quantification of trichothecene levels [10].

Results

Adhesion Molecule Expression on the Cell Surface of HBCEC and Astrocytes Induced by SH

HBCEC, astrocytes, and neurons were evaluated for the expression of inflammatory and apoptotic events from exposure to SH, lipopolysaccharide (LPS, a known inflammatory agent), and H_2O_2 (peroxide, a known oxidative-stress inducing agent). Negative controls were exposed to sterile, pyrogen-free water, and positive controls were exposed to 50 EU/ml LPS and 250 µM H_2O_2 in a volume of 20 µl. Lactate-dehydrogenase (LDH) concentrations were used as an indicator for cytotoxic events in cells exposed to conditions described in methods and additive

conditions (10 ng/ml SH+ LPS and 10 ng/ml SH + H_2O_2).

HBCEC and astrocytes were evaluated for the expression of adhesion molecule receptors (ICAM, VCAM, P/E selectin) expressed during inflammation. Figure 1a demonstrates immunofluorescent results from the expression of ICAM. Cells that expressed adhesion molecules receptors were bound by antibody (Ab) conjugated to the fluorescein isothiocynate (FITC). Density of fluorescence was used to quantitate the intensity of receptor expression for each sample group. A significant increase ($P < 0.05$) in the expression of ICAM on HBCEC cells exposed to SH 100, 1,000, and 10 ng/ml + H_2O_2 was demonstrated (similar results were demonstrated for VCAM and P/E selectin in HBCEC and astrocytes, Fig. 2a).

Permeability Across HBCEC from Exposure to Satratoxin H

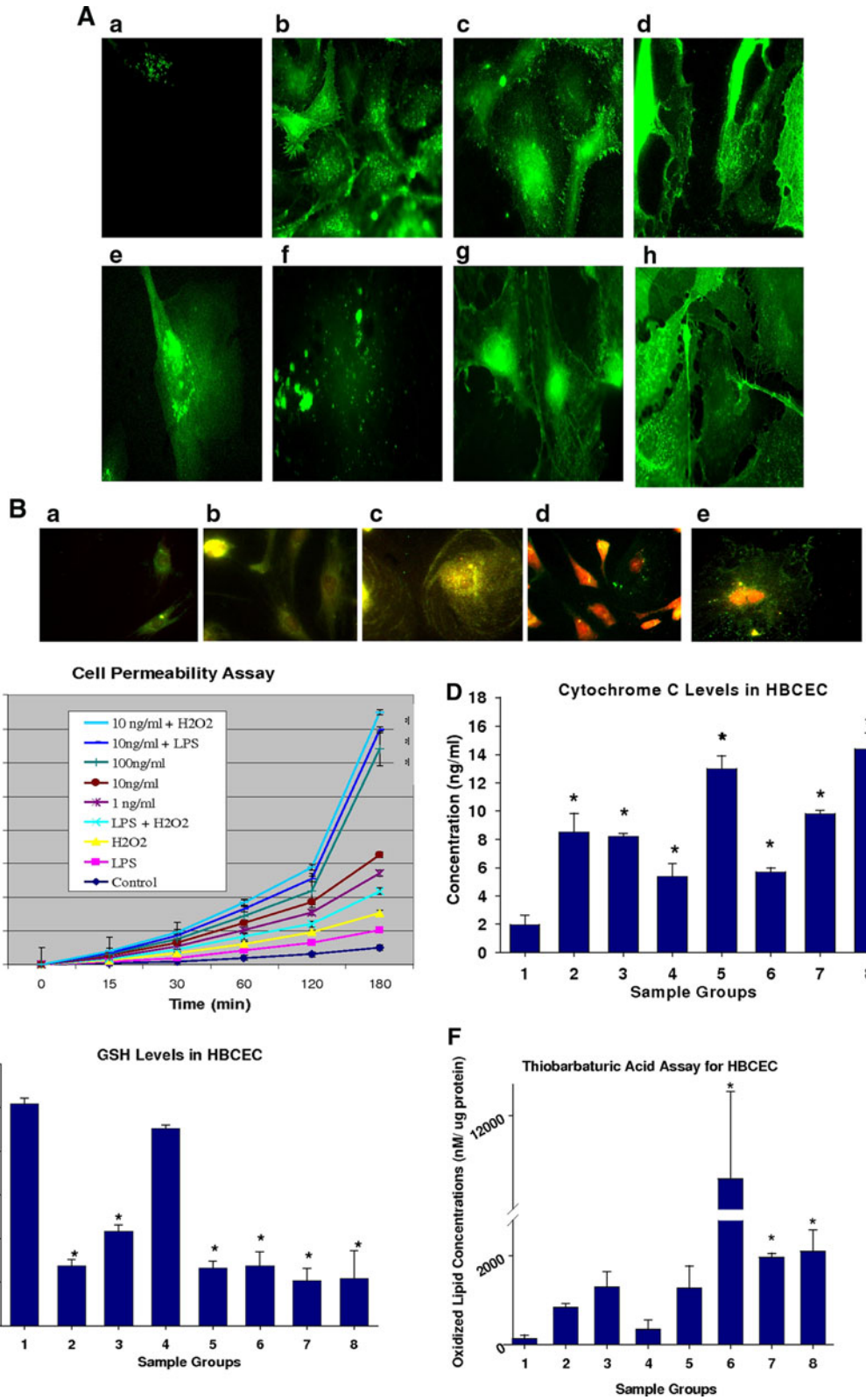
Some evidence of damage to the BBB is greater permeability across the barrier due to HBCEC cell shrinkage. Both the activation of inflammatory pathways and programmed cell death due to HBCEC exposure to toxic substances may increase the permeability of the BBB. Substances that are toxic to HBCEC may increase the permeability across the BBB due to programmed cell death. To evaluate if SH was able to induce HBCEC shrinkage in a BBB model, a monolayer of cells grown on 0.4-μm polycarbonate membranes in 24-well plates was exposed to ^{125}I-albumin and monitored every 15 min for the diffusion rate of albumin across the monolayer. There was a significantly greater rate ($P < 0.05$) of diffusion across cells exposed to 100 ng and 1,000 ng/ml of SH. A significant ($P < 0.05$) additive effect was observed in cells exposed to 10 ng/ml + LPS and 10 ng/ml + H_2O_2. These results are seen in Fig. 1c. Apoptotic-like events were observed in addition to inflammation.

Programmed Cell Death and Reactive Oxygen Species (ROS) in HBCEC and Astrocytes by SH

In the event of early-stage apoptosis, phosphatidylserine (PS) expressed on the inner cell membrane is flipped to the outer surface, an indication of apoptosis. In the detection assay, secondary Ab conjugated to FITC (green) binds to PS on the outer cell surface, in the event of apoptosis. Late stages of apoptosis consist of chromatin fragmentation and permeability of the nuclear membrane. During late-stage apoptosis events, propidium iodide (PI, red) binds to damaged chromatin material. These events are observed in Figs. 1b and 2b. Compared to the control (cells that received water), cells exposed to 100 ng/ml SH, 1,000 ng/ml SH, and LPS demonstrated early and late stages of apoptosis, whereas the control cells did not demonstrate PI in the nucleus of the cell. To further evaluate apoptosis, cytochrome C levels from cell extracts were evaluated using an ELISA method. Cytochrome C is maintained in the mitochondria of healthy cells. In the event of programmed cell death, cytochrome C is released from the mitochondria into the cytosol where it activates pathways associated with apoptosis. These results demonstrated that a significantly increased amount ($P < 0.05$) of cytochrome C was released from cells exposed to 10 ng/ml, 100 ng/ml, LPS, 10 ng/ml + LPS, and 10 ng/ml + H_2O_2. These results can be seen in Fig. 1d (similar results were observed in humans astrocytes and neurons; Figs. 2d and 3b, respectively).

During inflammatory responses and early apoptotic events, cells are under oxidative stress, which leads to the production of lipid radicals and lipid peroxidation. Previous studies have demonstrated that lipid radicals are able to inhibit anti-apoptotic genes, which allow a cell to enter into apoptosis. To further

Fig. 1 Inflammatory and oxidative stress responses of HBCEC to SH. **a** ICAM expression on HBCEC. *A* Control cells (H_20). *B* 10 ng/ml SH (SH). *C* 100 ng/ml SH. *D* 1,000 ng/ml SH. *E* 50 EU/ml. *F* 250 μM H_2O_2. *G* 10 ng/ml + LPS. *H* 10 ng/ml + H_2O_2. **b** Evaluation of HBCEC for apoptosis. Annexin V-FITC staining of PS and PI staining of DNA. *A* Control (H_20). *B* 10 ng/ml SH (SH). *C* 100 ng/ml SH. *D* 1,000 ng/ml SH. *E* LPS 50 EU/ml. **c** HBCEC evaluation for cell shrinkage and permeability. **d** Cytochrome C concentration from HBCEC. *1* Control cells (H_20). *2* 10 ng/ml SH. *3* 100 ng/ml SH. *4* 1,000 ng/ml SH. *5* 50 EU/ml. *6* 250 μM H_2O_2. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. * A significant increase ($P < 0.5$) compared to the control. **e** GSH concentrations from HBCEC. *1* Control cells (H_20). *2* 50 EU/ml LPS. *3* 250 μM H_2O_2. *4* 10 ng/ml SH (SH). *5* 100 ng/ml SH. *6* 1,000 ng/ml SH. *7* 10 ng/ml SH + LPS *8* 10 ng/ml SH + H_2O_2. * A significant increase ($P < 0.5$) compared to the control. **f** T-BARS assay for HBCEC. The degree of lipid oxidation induced in cells was quantitated by measuring the levels of lipid radicals from samples. *1* Control (H_2O). *2* LPS (50 EU/ml). *3* H_2O_2 (250 μM). *4* 10 ng/ml SH. *5* 100 ng/ml SH. *6* 1,000 ng/ml SH. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 532 nm. * A significant difference ($P < 0.05$) compared to the control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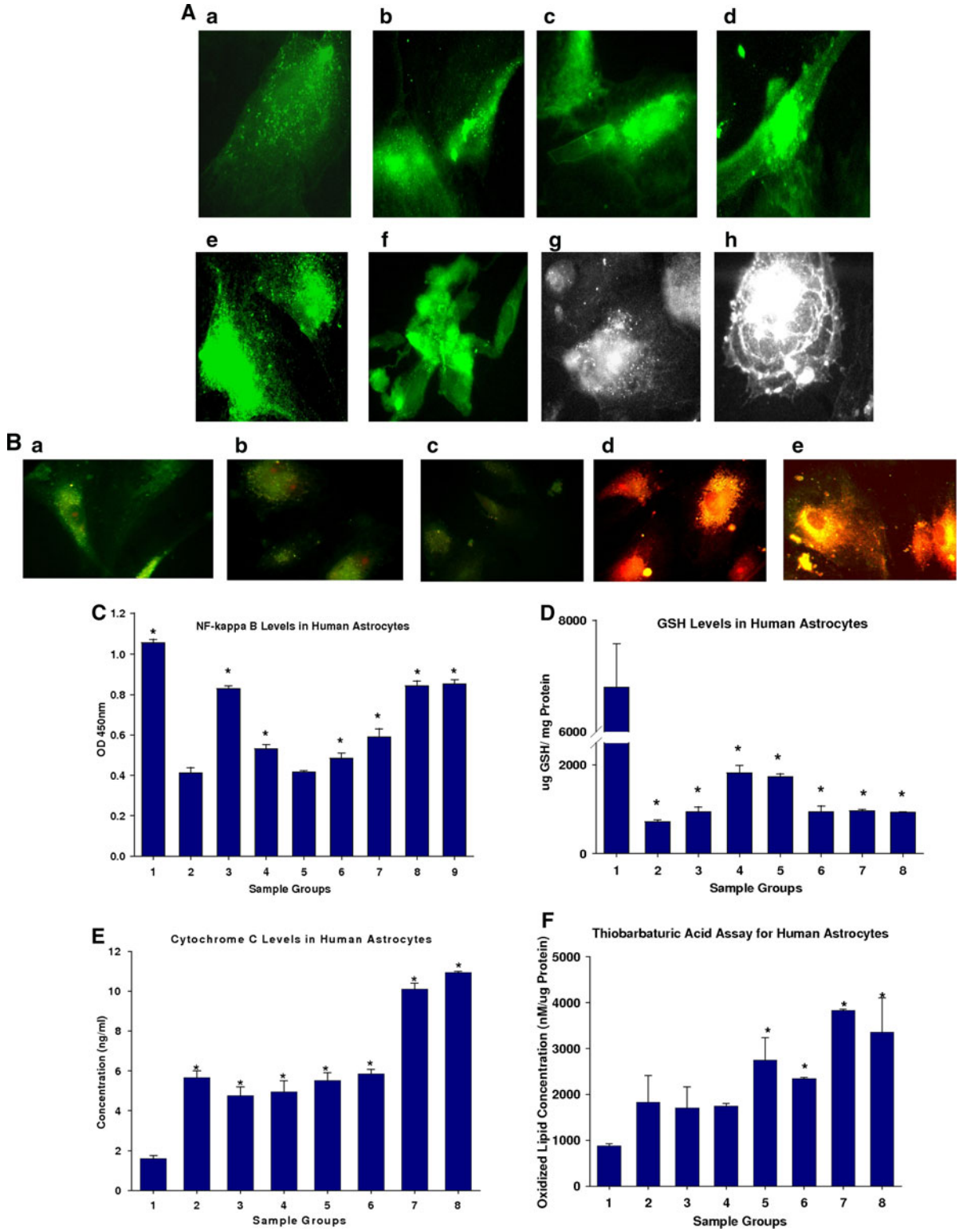
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NF-kappa B Levels in Human Astrocytes
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Cytochrome C Levels in Human Astrocytes
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Thiobarbaturic Acid Assay for Human Astrocytes
Oxidized Lipid Concentration (nM/ug Protein)
Sample Groups

◀ **Fig. 2** Human astrocytes response to SH exposure with inflammation and oxidative stress. **a** Densitometric evaluation of ICAM expression by human astrocytes. *1* Control cells (H_20). *2* 10 ng/ml SH (SH). *3* 100 ng/ml SH. *4* 1,000 ng/ml SH. *5* 50 EU/ml. *6* 250 μM H_2O_2. *7* 100 ng/ml SH + LPS. *8* 100 ng/ml SH + H_2O_2. * A significant increase ($P < 0.05$) compared to the control. **b** Evaluation of human astrocytes for apoptosis. Annexin V-FITC staining of PS and PI staining of DNA. *A* Control (H_20). *B* 10 ng/ml SH (SH). *C* 100 ng/ml SH. *D* 1,000 ng/ml SH. *E* 50 EU/ml LPS. **c** NF-κB concentrations from human astrocytes. *1* Positive control (HeLa cell extract) *2* Control cells (H_20). *3* 50 EU/ml LPS. *4* 250 μM H_2O_2. *5* 10 ng/ml SH (SH). *6* 100 ng/ml SH. *7* 1,000 ng/ml SH. *8* 100 ng/ml SH + LPS. *9* 100 ng/ml SH + H_2O_2. * A significant increase ($P < 0.5$) compared to the control. **d** Cytochrome C concentrations from human astrocytes. *1* Control cells (H_20). *2* 10 ng/ml SH. *3* 100 ng/ml SH. *4* 1,000 ng/ml SH. *5* 50 EU/ml LPS. *6* 250 μM H_2O_2. *7* 100 ng/ml SH + LPS. *8* 100 ng/ml SH+ H_2O_2. * A significant increase ($P < 0.05$) compared to the control. **e** GSH concentrations from human astrocytes. *1* Control cells (H_20). *2* 10 ng/ml SH (SH). *3* 100 ng/ml SH. *4* 1,000 ng/ml SH. *5* 50 EU/ml LPS. *6* 250 μM H_2O_2. *7* 100 ng/ml SH + LPS. *8* 100 ng/ml SH + H_2O_2. * A significant decrease ($P > 0.05$) compared to the control. **f** T-BARS assay for human astrocytes. The degree of lipid oxidation induced in cells was quantitated by measuring the levels of lipid radicals from samples. *1* Control (H_2O). *2* LPS (50 EU/ml). *3* H_2O_2 (250 μM). *4* 10 ng/ml SH. *5* 100 ng/ml SH. *6* 1,000 ng/ml SH. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 532 nm. * A significant difference ($P < 0.05$) compared to the control

evaluate apoptosis-associated events, indicators of oxidative stress were measured to evaluate cell damage.

During oxidative stress, glutathione (GSH) acts as a reducing agent against ROS such as lipid radicals and peroxides. However, if GSH levels in a cell are insufficient to compensate for the degree of oxidative stress, both apoptotic and inflammatory pathways are activated. In a healthy cell, GSH is unable to cross the nuclear membrane, but during oxidative stress the oxidized form of GSH, oxidized glutathione (GSSG), is able to cross this structure. GSSG is able to decrease the binding of p60/p65 complex of NF-κB to DNA, which reduces the pro-inflammatory cascade that is activated by NF-κB. To determine whether SH increased oxidative stress levels in HBCEC, a quantitative method was used to evaluate GSH concentrations present from cell extracts exposed to experimental conditions. The results demonstrated a significant decrease ($P > 0.05$) in the concentration of GSH (μg/ml) in HBCEC exposed to 100 ng/ml SH, 1,000 ng/ml SH, LPS, H_2O_2, 10 ng/ml + LPS, and 10 ng/ml + H_2O_2. These data are seen in Fig. 1e for HBCEC (similarly for astrocytes and neurons in Figs. 2e and 3c, respectively). The production of lipid peroxidation demonstrates the degree of oxidative stress induced on HBCECs. Results from the thiobarbituric acid assays (T-BARS) demonstrated that there was a significant increase ($P > 0.05$) in lipid peroxidation when cells were exposed to LPS, H_2O_2, moderate concentrations of SH (100 and 1,000 ng/ml), and additive conditions (10 ng/ml + LPS and 10 ng/ml + H_2O_2). These results can be seen in Fig. 1f for HBCEC (Fig. 2f and 3d demonstrate results for astrocytes and neurons, respectively).

The role of astrocytes in neural tissue is to produce protective compounds, through inflammation pathways in the event of disease. Immune cells are able to specifically amplify immune responses by the activation of transcription factors such as NF-κB. Using an ELISA method, the concentration of NF-κB was quantified as a measure of inflammation induced by SH. Results demonstrate a significant increase ($P < 0.05$) in the expression of NF-κB in cells exposed to 100 ng/ml SH, 1,000 ng/ml SH, LPS, 100 ng/ml SH + LPS, and 100 ng/ml + H_2O_2. These results can be seen in Fig. 2c.

Discussion

Diseases such as multiple sclerosis, Alzheimer's, rheumatoid arthritis, and systemic lupus erythematosus demonstrate inflammatory events leading to CNS damage [44, 46–48]. Upon exposure to low levels of a toxic compound or infectious agents, cells are able to produce low levels of inflammation and apoptotic events simultaneously [49–52]. During neuroinflammation, neurons tend to be damaged by immune responses, and the reaction of astrocytes and endothelial cells can amplify CNS damage [44, 47, 48, 51, 52].

As demonstrated by previous studies through Calderon-Garciduenas et al. in a in vivo canine model and rodent studies by other research groups fungal toxins and small fungal fragments within indoor environments can lead to permanent CNS tissue damage (including damage to the BBB) [13, 14, 17, 29, 30]. If HBCECs are damaged or cell shrinkage occurs, as evidenced by these data through trichothecenes such as SH, these mycotoxins or

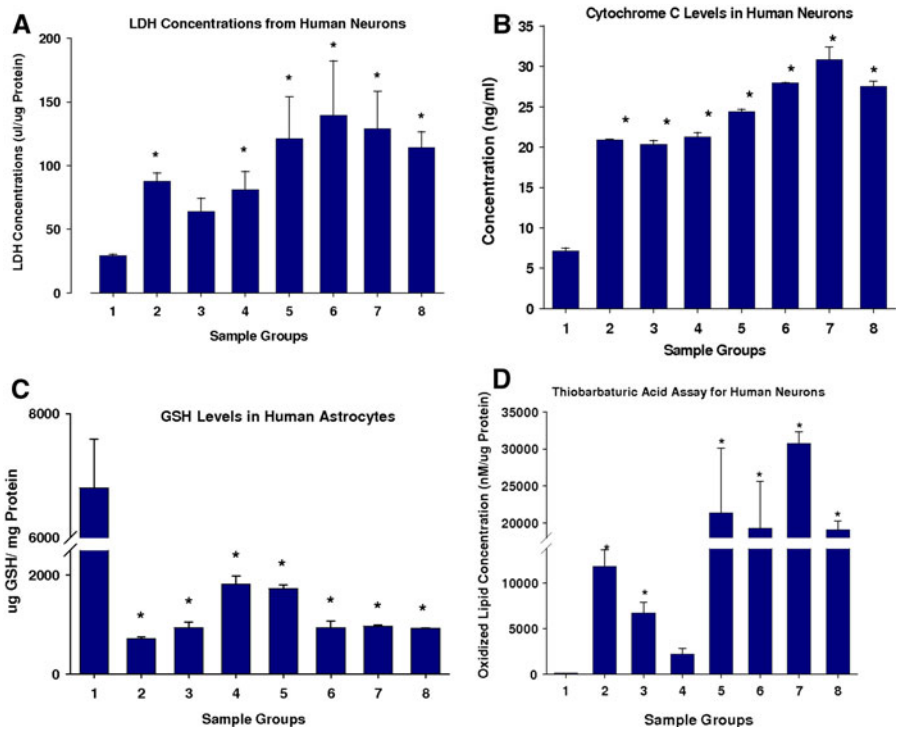

Fig. 3 Oxidative stress response and apoptosis events in human neurons, due to SH exposure. **a** LDH assay for human neurons. *1* Control (H_2O). *2* LPS (50 EU/ml). *3* H_2O_2 (250 μM). *4* 10 ng/ml SH. *5* 100 ng/ml SH. *6* 1,000 ng/ml SH. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 340 nm. * A significant difference ($P < 0.05$) compared to the control. **b** Cytochrome C concentration from human neurons. *1* Control cells (H_2O). *2* 10 ng/ml SH. *3* 100 ng/ml SH. *4* 1,000 ng/ml SH. *5* 50 EU/ml. *6* 250 μM H_2O_2. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. * A significant increase ($P < 0.05$) compared to the control. **c** GSH concentrations from human neurons. *1* Control cells (H_2O). *2* 10 ng/ml SH (SH). *3* 100 ng/ml SH. *4* 1,000 ng/ml SH. *5* 50 EU/ml. *6* 250 μM H_2O_2. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. * A significant decrease ($P > 0.05$) compared to the control. **d** T-BARS assay for human neurons. The degree of lipid oxidation induced in cells was quantitated by measuring the levels of lipid radicals from samples. *1* Control (H_2O). *2* LPS (50 EU/ml). *3* H_2O_2 (250 μM). *4* 10 ng/ml SH. *5* 100 ng/ml SH. *6* 1,000 ng/ml SH. *7* 10 ng/ml SH + LPS. *8* 10 ng/ml SH + H_2O_2. Samples were read spectrophotometrically at 532 nm. * A significant difference ($P < 0.05$) compared to the control

similar toxic agents could pass through the BBB and come into contact with astrocytes or neurons, causing further cellular damage.

Since astrocytes behave as macrophages, the production of ROS, cytokines, chemokines, and other pro-inflammatory agents released in response to the additive effects of moderate doses of SH in the presence of other agents (e.g., LPS) or oxidative stress (H_2O_2) could overwhelm astrocytes and lead to programmed cell death (apoptosis-associated events). The release of these cell-stimulants into the neural tissues could activate HBCECs and neurons to enter programmed cell death or produce increased inflammatory events. This could lead to devastating events, particularly in neurons, since neurons are dependent on astrocytes for protective signals to delay apoptosis.

Programmed cell death in neurons as demonstrated by these and other studies can lead to the permanent neurological damage, experienced by individuals exposed to spore-free particles carrying mycotoxins indoors, as neurological cells are not fully regenerated in adult neural tissues [4, 13–15, 17, 30].

The activation of these inflammatory and apoptotic pathways on a chronic basis could shift the homeostasis of these cells, leading to degradation of the BBB. The loss of activity of the BBB due to SH exposure could potentially amplify the damage to neural tissues in multiple ways. If an individual is chronically exposed to low to moderate levels of

airborne trichothecenes like SH, the risk of neurological damage from constant activation of inflammatory/apoptotic-like pathways in the body could increase [35, 36].

These results suggest that low to moderate levels of SH are able to induce inflammatory and apoptotic pathways that amplify cellular damage by the continuous activation of these biological pathways. The presence of lipid peroxidation in cells exposed to moderate concentrations of SH and additive conditions (due to endotoxin) demonstrates the ability of the mycotoxins to amplify cellular damage through the indirect production of lipid radicals and other ROS.

This study suggests that individuals exposed to SH and microbial organisms resulting in a chronic immune response (inflammation and oxidative stress) could have increased sensitivity to these agents, leading to neural damage, further supporting previous in vivo studies demonstrating CNS tissue damage via inhalation of fungal toxins [30]. These results demonstrate that regardless of whether the neurons were exposed to SH alone or under additive effects, the sensitivity of the neurons to these compounds is high and neurological system cell damage can occur from SH exposure. In addition, these data demonstrate that constant activation of inflammatory and apoptotic pathways at low levels, amplifies the devastation and leads to neurological cell damage from indirect events triggered by the presence of a trichothecene mycotoxin. From this study and others, we show that neurological system cell damage from exposure to mycotoxins is a potential public health threat for occupants of water-damaged buildings. The public health threat posed by exposure to mycotoxin-producing fungi in the indoor environment is not fully understood. However, those who venture back to the areas affected by severe water damage, like hurricanes Katrina and Rita should be aware of the potential for exposure to mycotoxins and the subsequent risk for neurological system cell damage [3, 13, 33, 34].

Experimental Procedures

Satratoxin H Purification Methods

Sixteen 2-l sterile, autoclaved Erlenmeyer flasks (Pyrex, Fisher Scientific, Houston, TX) were filled with 250 g of Uncle Ben's long-grain-enriched parboiled rice and 125 ml of sterile pyrogen-free water. Each flask was inoculated with 50 ml of *S. chartarum* growth from 14-day cultures grown on potato-dextrose agar plates (PDA). To produce maximum sporulation and mycotoxin production, a strain known to be a toxin producer was graciously provided by Dr. Bruce Jarvis at the University of Maryland, (strain number 29-16-6). The flasks were incubated for 6 weeks in a disinfected, negative-pressure chamber with high efficiency particulate air (HEPA) filtration. The rice cultures were allowed to incubate at room temperature (RT), 25°C, with a relative humidity level in the chamber of 43% with periodic shaking every 2 days to prevent the rice from clumping.

After the 6-week growth period, each culture was washed and agitated in a 1:1 chloroform/methanol (Fisher Scientific, Houston, TX) solution at 4°C overnight with constant shaking at 3,000 rpm. The black chloroform/methanol solution recovered from the washing process was collected into holding containers. Each rice culture was washed a second time with 150 ml of methanol following the same procedure previously described.

A total of 10 l of harvested solution was collected. The solution was vacuum filtered through a Whatmann's number 1 filter to remove large debris collected from the rice cultures. Next, aliquots of the harvested solution were vacuum filtered through a 0.45-μm filter and subsequently 0.22 μm (Millipore, Fisher Scientific). At this stage of the filtration process, a large amount of lipids would collect on the surface of the filters, only allowing 150 ml of solution to be collected from one filter.

The filtered solution was rotary evaporated to remove the solvent and collect crude toxin extract. The boiling point (BP) of chloroform is between 61°C and 62°C and the BP for methanol is 64.7°C. The rotary evaporator (Büchi Model, Brinkman Instruments, Westbury, NY) was kept under vacuum pressure with cool water (2.4–4.0°C) flowing through the condenser. Approximately, 210 ml of the filtered solution was evaporated and a golden-brown solution remaining in the flask suspended in methanol.

The crude extracts were purified by column chromatography methods. Samples of the crude extract were dried overnight on 5.0 g of Florisil (100–200 mesh) (Fisher Scientific, Fair Lawn, NJ). Purified extracts were collected by solid-phase extraction

methods. The solid phase was re-purified with high-performance liquid chromatography (HPLC), using an 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) equipped with an ultraviolet–visible (UV/Vis) diode array detector [32]. A 400 (250 + 150)-mm Eclipse C8 analytical column plus a 12.5-mm guard column set at 40°C was used in the system [32]. The flow rate was set at 1.0 ml/min [32]. Crude toxin samples in methanol were run in a mobile phase in which the gradient changed from 35% acetonitirile in water to 80% in 18 min [32]. Samples were detected at 260 nm and analyzed using Chemstation software (Agilent Technologies, Palo Alto, CA). Standards of SH were kindly donated by B.B. Jarvis. SH samples collected through HPLC methods in our laboratory were compared with B.B. Jarvis' sample and further verified with UV spectra analysis compared to previous descriptions in the literature for SH [32, 53–55]. To further verify that the purified samples collected were SH; samples were analyzed with columns of different polarity to verify individual peaks.

Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, is a common contaminant in the environment. To prevent LPS-induced activation of cell cultures, samples of SH were tested for contamination levels. A QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) Assay (Cambrex-Biowhitakker, Walkersville, MA) was used to determine LPS concentrations and read photometrically at 405–410 nm. The concentration of endotoxin in a sample was calculated from a standard curve developed with an *Escherichia coli* LPS sample provided.

Known concentrations of SH were placed in 2.0-ml glass vials (Agilent Technologies, Palo Alto, CA) and heated in a gravity oven (Gravity Oven Lindberg/Blue M, Asheville, NC) at 180°C for 4 h. These heated SH samples were suspended in pyrogen-free water, supplied in the QCL-1000 kit to a concentration of 1 mg/ml and tested through HPLC analysis which demonstrated that the SH was not present in the vials. This showed that the compound was unable to withstand prolonged heat exposure at 180°C for 4 h. Following these results, careful procedures were undertaken to transfer purified samples of SH from the HPLC into baked, sterile glassware. The standard level of endotoxin contamination below which an inflammatory response does not occur is 250 pg/ml (0.25 EU/ml). The purified SH samples had a mean endotoxin concentration of 0.075 EU/ml (±0.011 S.E.). Since the concentration of endotoxin in SH samples was below the acceptable level of contamination, LPS contamination was not a contributing factor in our experimental results.

Cell Culture Methods

HBCEC, astrocytes, and progenitor neuronal cells (Cambrex-Biowhittaker, Walkersville, MD), cell culture medium, and growth factors were purchased from Cambrex-Biowhittaker, Clonetics Division (Walkersville, Maryland). Five $\times 10^5$ cells were cultured in 25 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). Cells were maintained in 5.0 ml of endothelial cell growth medium (EGM-2MV) with the following growth factors: 2% (vol/vol) fetal bovine serum (FBS), human recombinant growth factor (10 ng/ml), EC growth supplement (12 μg/ml), hydrocortisone (1.0 μg/ml), and gentamicin (50 μg/ml) incubated with 5% CO_2 at 37°C. The confluent cells were sub-cultured on unsiliconized glass cover slips (25 mm × 25 mm) [52]. These glass cover slips were treated with a series of ethanol and water washes to remove the silicon coating, according to procedures published by Simoni et al. [52]. Two coverslips were seeded per well in a six-well plate (Corning Inc.). In addition to coverslips, the cells were subcultured in a series of 24-well plates until confluent (Corning Inc.). Experiments were conducted on cells from either a fourth or fifth passage. Cell passages were performed using the trypsin method recommended by Cambrex-Biowhittaker, Walkersville, MD. The cell lines were tested by the supplier for human immunodeficiency virus, hepatitis, *Mycoplasma*, bacteria, yeast, fungi, and smooth muscle α-actin expression. The results of these tests were presented in Clonetics Certificate of Analysis.

Prior to experimentation with SH, if endotoxin removal was required, an affinity chromatography method was utilized according to the manufacturer protocols for Detoxi-Gel Endotoxin Removal Gel AffinityPak Prepacked Columns (Pierce, Rockford, IL), followed by further evaluation using the QCL-1000 assay (as previously described).

Previous in vitro studies have demonstrated that concentrations of 10 ng/ml of macrocyclic trichothecenes were able to induce significant increases in cytokine synthesis and other cellular processes [40]. In the experiments here, 1, 10, 100, 1000, and

5,000 ng/ml concentrations of SH were evaluated. Prior to the addition of the compounds, the media in the 6- and 12-well plates were replaced with fresh medium. The samples were then suspended in pyrogen-free, sterile water (Cambrex-Biowhittaker, Inc., Walkersville, MD) and incubated with SH for 18 h. Control cells received an equal volume of water. A second subset of cells were exposed to the concentrations of SH previously mentioned with the addition of 250 μM H_2O_2 or 50 EU/ml LPS to induce an inflammatory response.

A general cytotoxicity assay was conducted to determine which sample groups were able to produce cytotoxic effects in cells. LDH concentrations were used to determine the cytotoxic effects of SH on HBCEC, astrocytes, and neuronal cells. A commercial assay, LDH (DG-1340-UV) concentration assay (Sigma Diagnostics), was utilized to determine the concentration of LDH released by cells into the culture medium after an 18-h incubation period. The procedures described in the assay were followed to determine the concentration of LDH released by cells due to cellular damage. Samples were read spectrophotometrically at 340 nm. The intent of this assay is to reflect potential cellular damage induced by SH.

Immunocytochemistry

A series of immunofluorescent studies were conducted to evaluate inflammatory events in HBCEC due to SH exposure. Cells grown on coverslips as described previously were washed with cold phosphate-buffered saline (PBS) twice, followed by exposure to 3% formaldehyde in PBS for 20 min at RT [52]. The cells were exposed to cold methanol, followed by a series of four washings with PBS for a 30-min period to permeabilize the cell membrane [52]. These cells were then blocked against nonspecific antibody binding with 1% bovine serum albumin (BSA) (Sigma Chemical Co.). Cells were exposed to monoclonal antibody against human ICAM-1, VCAM-1, and P/E selectin for 1 h at 37°C (R&D Systems). The wells were washed five times with PBS, followed by a 1-h incubation with secondary antibody anti-mouse, FITC conjugated F $(ab')_2$ (Boehringer Mannheim, Co., Biochemical Products, Indianapolis, IN). Following a 30-min incubation period, coverslips were mounted onto slides, and later evaluated microscopically at 60× under oil-emersion. This procedure demonstrated if SH was able to induce the expression of adhesion molecule receptors. To quantitate the degree of fluorescence, photographs of cells were scanned and evaluated with Scan Analysis Software (Biosoft, Cambridge, UK). This procedure measured the density of fluorescence in each picture and reported the results in units of luminescence; total area under a peak was used to determine the degree of fluorescence. Photographs from duplicate coverslips under the same experimental conditions conducted in triplicate for each of the adhesion molecule receptors were evaluated.

BBB-Model Permeability Assay

To evaluate whether SH is able to increase permeability across the BBB model, the passage of ^{125}I-albumin (Diagnostic Products Corp., Los Angeles, CA) was evaluated. Cells were cultured in 75 cm^2 tissue culture flasks with EGM-2MV (Cambrex-Biowhittaker, Walkersville, MD), in humidified conditions with 5% CO_2, until they reached confluence, and were subcultured in 24-well plates tissue culture treated, 0.4-μm polycarbonate membrane cell culture devices (Whatman Inc., Clifton, NJ). Radioactive-albumin (^{125}I) was incubated with a monolayer of cells exposed grown on 0.4-μm polycarbonate membranes in 24-well culture plates to control conditions (pyrogen-free water), LPS, H_2O_2, and SH. ^{125}I-albumin from supernatant was monitored every 15 min for 3 h for the diffusion rate of albumin across the monolayer. Radioactivity was measured using a scintillation counter as counts per unit (CPU).

Cell Death and Apoptosis Assays

HBCEC or astrocytes cultured on unsiliconized glass coverslips were evaluated after exposure to SH and control conditions for apoptosis utilizing Apoptosis Detection Kit Annexin V-FITC (APO-AP) (Sigma, St. Louis, MO). Early apoptotic events were determined by monoclonal antibody (anti-PS fluorescein conjugate) binding to PS translocated from the inner cell membrane to the outer membrane. Late-stage apoptosis was evaluated by PI binding to DNA fragments through translocation to the nucleus. The detection of immunofluorescent staining was evaluated microscopically (Olympus-IX71 Confocal

Microscope, Leeds Inst. Inc., Irving, TX). The software program used to view fluorescence was Metamorph® (Universal Imaging Corp., Dowington, PA). These assays demonstrated the concentration of SH able to induce apoptosis in HBCEC or astrocytes.

To quantify the degree of apoptosis in cells, an ELISA was used to determine the levels of cytochrome C from cellular extracts. The ELISA for the evaluation of cytochrome C was conducted according to protocols described in the manual provided by R&D Systems (Minneapolis, MN).

In the experiments conducted 1, 10, 100, 1000, and 5,000 ng/ml concentrations of SH were evaluated. Prior to the addition of the compounds, the media in the 6 and 12-well plates were replaced with fresh medium. The samples were then suspended in pyrogen-free, sterile saline solution (Cambrex-Biowhittaker, Inc., Walkersville, MD) and incubated with SH for 18 h. Control cells received an equal volume of water. A second subset of cells were exposed to the concentrations of SH previously mentioned with the addition of 250 μM H_2O_2 and 50 EU/ml LPS to induce an inflammatory response.

NF-κB Assay

To evaluate the expression of NF-κB in astrocytes, cell extracts from astrocytes exposed to previously described experimental conditions were measured using an ELISA method. The methods used to conduct this assay were performed according to the procedures outlined by the manufacturers of the ELISA kit (R&D Systems Inc.). The nuclear translocation and DNA binding of NF- κB were assayed in nuclear cell extracts using a TransAM™ NF-κB p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA). This method is the first ELISA-based kit to detect and quantify NF-κB activation that contains a 96-well plate on which there is an immobilized oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′). The whole-cell extracts were obtained from living cells using complete lysis buffer that contained dithothreitol (DTT) and a protease inhibitor cocktail supplied by the manufacturer. The complete binding buffer was supplemented with DTT and herring sperm DNA. Results were expressed in OD at 450 nm per 2.5 μg of whole-cell extract. The HeLa whole-cell extracts, provided by the manufacturer, were used as a positive control for NF-κB activation and DNA binding. This colorimetric assay provided quantitative evidence of inflammatory pathway activation in astrocytes.

Oxidative Stress Assays and Concentration of ROS

In these experiments, the presence of oxidative stress was evaluated by measuring lipid hyperperoxides (LOOH) and reduced glutathione (GSH) levels according to established methods [49–52].

Intracellular GSH levels were measured from cell homogenates [51]. The reaction of GSH with 5, 5′ dithiobis and sulfhydryl compounds leads to a color change producing a yellow pigment. The samples were read spectrophotometrically at 412 nm (Sigma Chemical Co., St Louis, MO). Results were compared to a standard curve using GSH in nanomoles per milligram of protein. Protein concentrations for the cell homogenate fractions were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Lipid hydroperoxides were evaluated in cell homogenate fractions with a previously established method [32, 54, 55]. This assay is able to directly measure the LOOH concentration. Samples were mixed with sodium dodecyl sulfate (SDS)-acetate buffer, pH 3.5, and an aqueous solution of T-BAR acid. The reaction mixture was heated to 95°C for 90 min. After cooling, the red-colored complex was extracted with *n*-butanol-pyridine [49–51]. The data were measured spectrophotometrically at 532 nm and compared to a standard curve [49–51]. Data were expressed in nanomoles of LOOH per microgram of cells [49–51].

Statistical Analysis Methods

Statistical analysis ($\alpha = 0.05$) was performed using Sigma Stat, to analyze the data using one-way analysis of variance (ANOVA) of each experimental group with the controls. Normality of the data was determined using a Kolmogorov–Smirnov normality test. If data did not meet normality, a nonparametric Kruskal–Wallis ANOVA was applied. If data were normal, and ANOVA demonstrated significance, a post hoc Tukey test was used to make multiple comparisons to determine which experimental groups demonstrated significance compared to controls and

between experimental groups. Results were graphed using Sigma Plot.

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References

1. Manuel J. In Katrina's wake. Environ Health Perspect. 2006;114:A32–9.
2. Solomon GM, Hjelmroos-Koski M, Rotkin-Ellman M, Hammond SK. Airborne mold and endotoxin concentrations in New Orleans, Louisiana, after flooding, October through November 2005. Environ Health Perspect. 2006;114:1381–6.
3. Reponen T, et al. Fungal fragments in moldy houses: a field study in homes in New Orleans and Southern Ohio. Atmos Environ. 2007;41(37):8140–9.
4. Bloom E, et al. Molds and mycotoxins in indoor environments—a survey in water-damaged buildings. J Occup Environ Hyg. 2009;6(11):671–8.
5. Adhikari A, et al. Aerosolization of fungi, (1–>3)-beta-D glucan, and endotoxin from flood-affected materials collected in New Orleans homes. Environ Res. 2009;109(3): 215–24.
6. Cooley JD, Wong WC, Jumper CA, Straus DC. Correlation between the prevalence of certain fungi and sick building syndrome. Occup Environ Med. 1998;55:579–84.
7. Cooley JD. The role of *Penicillium chrysogenum* conidia in sick building syndrome and an asthma-like animal model. A dissertation in Medical Microbiology and Immunology. Lubbock: Texas Tech University Health Sciences Center; 1999.
8. Brightman HS, Moss N. Sick building syndrome studies and the compilation of normative and comparative values. In: Samet JM, Spengler JD, McCarthy JF, editors. Indoor air quality handbook, vol. 3. New York: McGraw-Hill; 2000.
9. Finnegan MJ, Pickering CAC, Burge PS. The sick building syndrome—prevalence studies. BMJ. 1984;289:1573–5.
10. I.O.M (U.S.). Damp indoor spaces and health. In: Committee on damp indoor spaces and health. Washington, DC: National Academies Press; 2004.
11. Hodgson M. Field studies on the sick building syndrome. Ann N Y Acad Sci. 1992;641:21–36.
12. Teichman KY. Indoor air-quality research needs. Occup Med State Art Rev. 1995;10:217–27.
13. Calderon-Garciduenas L, et al. Air pollution and brain damage. Toxicol Pathol. 2002;30(3):373–89.
14. Calderon-Garciduenas L, et al. DNA damage in nasal and brain tissues of canines exposed to air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration. Toxicol Pathol. 2003;31(5):524–38.
15. Edmondson DA, et al. Immune response among patients exposed to molds. Int J Mol Sci. 2009;10(12):5471–84.
16. Johanning E, Biagini R, Hull D, Morey P, Jarvis B, Landsbergis P. Health and immunology study following exposure to toxigenic fungi (*Stachybotrys chartarum*) in a water-damaged office environment. Int Arch Occup Environ Health. 1996;68:207–18.
17. Kilburn KH, Thrasher JD, Immers NB. Do terbutaline- and mold-associated impairments of the brain and lung relate to autism? Toxicol Ind Health. 2009;25(9–10): 703–10.
18. Fung F, Clark R, Williams S. *Stachybotrys*, a mycotoxin-producing fungus of increasing toxicologic importance. J Toxicol Clin Toxicol. 1998;36:79–86.
19. Bata A, Harrach B, Ujszaszi K, Kistamas A, Lasztity R. Macrocyclic trichothecene toxins produced by *Stachybotrys atra* strains isolated in middle Europe. Appl Environ Microbiol. 1985;49:678–81.
20. Harrach B, Bata A, Bajmocy E, Benko M. Isolation of satratoxins from the bedding straw of a sheep flock with fatal stachybotrytoxicosis. Appl Environ Microbiol. 1983;45: 1419–22.
21. Harrach B, Mirocha CJ, Pathre SV, Palyusik M. Macrocyclic trichothecene toxins produced by a strain of *Stachybotrys atra* from Hungary. Appl Environ Microbiol. 1981;41:1428–32.
22. Harrach B, Bata A. Macrocyclic trichothecene *Stachybotrys* toxins isolated from field samples. In: Proceedings of the 5th international IUPAC sympsosium on mycotoxins and phycotoxins. Vienna; 1982.
23. Larrañaga MD (2004) The capability of a solid sorbent desiccant unit at removing selected indoor air quality-related microorganisms from the air. A dissertation in industrial engineering. Lubbock: Texas Tech University.
24. Bernton HS. Asthma due to a mold—*Aspergillus fumigatus*. J Am Med Assoc. 1930;95:189–92.
25. Seo SC, et al. Aerosolization of particulate (1–>3)-beta-D-glucan from moldy materials. Appl Environ Microbiol. 2008;74(3):585–93.
26. Peters A, Veronesi B, Calderon-Garciduenas L, Gehr P, Chen LC, Geiser M, Reed W, Rothen-Rutishauser B, Schurch S, Schulz H. Translocation and potential neurological effects of fine and ultrafine particles a critical update. Part Fibre Toxicol. 2006;3(13):1–13.
27. Hildebrandt K, et al. Short-term effects of air pollution: a panel study of blood markers in patients with chronic pulmonary disease. Part Fibre Toxicol. 2009;6:13.
28. Rafal GL. Filamentous microorganisms and their fragments in indoor air—a review. Ann Agric Environ Med. 2004;11:185–97.
29. Brasel TL, et al. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins in the indoor environment. Appl Environ Microbiol. 2005;71(11): 7376–88.
30. Thrasher JD, Crawley S. The biocontaminants and complexity of damp indoor spaces: more than what meets the eyes. Toxicol Ind Health. 2009;25(9–10):583–615.
31. Brasel TL, Martin JM, Carriker CG, Wilson SC, Straus DC. Detection of airborne *Stachybotrys chartarum* macrocyclic trichothecene mycotoxins in the indoor environment. Appl Environ Microbiol. 2005;71:7376–88.
32. Karunasena E, Cooley JD, Douglas DR, Straus DC. Protein translation inhibition by *Stachybotrys chartarum* conidia

with and without the mycotoxin containing polysaccharide matrix. Mycopathologia. 2004;158:87–97.
33. Rosen J, Heseltine E. WHO guidelines for indoor air quality: Dampness and Mould. Copenhagen: WHO; 2009. p. 1–228.
34. Office, U.S.G.A. Indoor mold: better coordination of research on health effects and more consistent guidance would improve federal efforts, U.S.G.A. Office, Editor. United States Government Accountability Office; 2008. p. 1–60.
35. Islam Z, Harkema JR, Pestka JJ. Satratoxin G from the black mold *Stachybotrys chartarum* evokes olfactory sensory neuron loss and inflammation in the murine nose and brain. Environ Health Perspect. 2006;114:1099–107.
36. Islam Z, Amuzie CJ, Harkema JR, Pestka JJ. Neurotoxicity and inflammation in the nasal airways of mice exposed to the macrocyclic trichothecene mycotoxin roridin A: kinetics and potentiation by bacterial lipopolysaccharide coexposure. Toxicol Sci. 2007;98:526–41.
37. Sharma RP, Kim YW editors. Macrocyclic trichothecenes. In Sharma RP, Salunkhe DK editors. Mycotoxins and phytoalexins in human and animal health. CRC Press: Boca Raton; 1991.
38. Croft WA, Jarvis BB, Yatawara CS. Airborne outbreak of trichothecene toxicosis. Atmos Environ. 1986;20:549–52.
39. Bata A, Harrach B, Ujszaszi K, Kistamas A, Lasztity R. Macrocyclic trichothecene toxins produced by *Stachybotrys atra* strains isolated in middle Europe. Appl Environ Microbiol. 1985;49:678–81.
40. Jarvis BB editor. Macrocyclic trichothecenes. In: Sharma RP, Salunkhe DK editors. Mycotoxins and phytoalexins in human and animal health. Boca Raton: CRC Press; 1991.
41. Nikulin M, Pasanen AL, Berg S, Hintikka EL. *Stachybotrys atra* growth and toxin production in some building-materials and fodder under different relative humidities. Appl Environ Microbiol. 1994;60:3421–4.
42. Anyanwu E, Campbell AW, High W. Brainstem auditory evoked response in adolescents with acoustic mycotic neuroma due to environmental exposure to toxic molds. Int J Adolesc Med Health. 2002;14:67–76.
43. Lees-Haley PR. Toxic mold and mycotoxins in neurotoxicity cases: *Stachybotrys*, *Fusarium*, *Trichoderma*, *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria*, *Trichothecenes*. Psycholol Rep. 2003;93:561–84.
44. Campbell IL. Neuropathogenic actions of cytokines assessed in transgenic mice. Int J Dev Neurosci. 1995; 13:275–84.
45. Dong W, Azconda-Olivera JI, Brooks KH, Linz JE, Pestka JJ. Elevated gene expression and production of interleukins 2, 4, 5, and 6 during exposure to vomitoxin (deoxynivalenol) and cycloheximide in the EL-4 thymoma. Toxicol Appl Pharmacol. 1994;127:282–90.
46. Ballabh P, Braun A, Nedergaard M. The blood-brain barrier: an overview—structure, regulation, and clinical implications. Neurobiol Dis. 2004;16:1–13.
47. Chavarria A, Alcocer-Varela J. Is damage in the central nervous system due to inflammation? Autoimmun Rev. 2004;3:251–60.
48. Ceccatelli S, Tamm C, Sleeper E, Orrenius S. Neural stem cells and cell death. In: 41st congress of the European-societies-of-toxicology. Florence, Italy; 2003.
49. Simoni J, Simoni G, Garcia EL, Prien SD, Tran RM, Feola M, Shires GT. Protective effect of selenium on hemoglobin mediated lipid peroxidation in vivo. Artif Cells Blood Substit Immobil Biotechnol. 1995;23:469–86.
50. Simoni J, Simoni G, Wesson D, Griswold JA, Feola M. A novel hemoglobin-adenosine-glutathione based blood substitute: evaluation of its effects on human blood ex vivo. Am Soc Art Intern Organs J. 2000;46:679–92.
51. Simoni J, Simoni G, Martinez-Zaguilan R, Wesson DE, Lox CD, Prien SD, Kumar RV. Improved blood substitute: evaluation of its effects on human endothelial cells. Am Soc Art Intern Organs J. 1998;44:356–67.
52. Simoni J, Simoni G, Lox CD, Prien SD, Shires GT. Evidence for the direct inhibition of endothelin-1 secretion by hemoglobin in human endothelial cells. Am Soc Art Intern Organs J. 1995;41:641–51.
53. Wannemacher, RW, Wiener SL, editors. Textbook of military medicine, part I. Warfare, weaponry, and the casualty, medical aspects of chemical and biological warfare. In: Bellamy RF, Zajtchuk R editors. Trichothecene mycotoxins. Office of the Surgeon General: Washington, DC; 1997.
54. Hinkley SF, Jarvis BB. Chromatographic methods for *Stachybotrys* toxins. Methods Mol Biol. 2000;157:173–94.
55. Jarvis BB, Salemme J, Morais A. *Stachybotrys* toxins. Nat Toxins. 1995;3:10–6.